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(54) [Title of the Invention] Monoclonal Antibodies, a Hybridoma that Produces Them and a Method for the Production of Said Antibodies

(57) [Abstract]

[Structure] Monoclonal antibodies that specifically recognize human integrin β_7, a hybridoma that produces them and a method for the production of said antibodies.

[Effect] Human integrin β_7 in human tissues can easily be detected by using the monoclonal antibodies of this invention. In addition, human integrin β_7 can be separated and purified from human tissues by using the monoclonal antibodies of this invention. Consequently, the monoclonal antibodies of this invention are used as reagents for the detection of human integrin β_7 and as a reagent for separating and purifying it. Moreover, because the monoclonal antibodies of this invention control adhesion of B-cell lymphocytes (RPMI8866) to fibronectin, it is related to cell adhesion proteins such as fibronectin. Its application as a therapeutic agent for inflammatory diseases can be anticipated.

[Claims]

[Claim 1] Monoclonal antibodies that specifically recognize human integrin β_7.

[Claim 2] A hybridoma that produces the antibodies described in Claim 1.

[Claim 3] A method for the production of monoclonal antibodies that specifically recognize human integrin β_7 characterized in that cells that express human integrin α_4 but that do not express human integrin β_1 are selected by the flow-cytometry analysis method from human lymphocytes selected from human memory T-cells, human monocytes and B-cell lymphoma using anti-human integrin α_4 antibodies and anti-human integrin β_1 antibodies, after which a solubilized solution of said cells is introduced into an immunoaffinity column in which anti-human integrin α_4 antibodies are immobilized, with a human integrin α_4 β_7 heterodimer being obtained, which heterodimer is then used as an antigen to immunize a small mammal, after which its spleen is excised and antibody-producing spleen cells are prepared, said antibody producing spleen cells and myeloma cells then being subjected to cell fusion to prepare a hybridoma, monoclonal antibodies that said hybridoma produces being screened by flow-cytometry analysis, a hybridoma that does not recognize human integrin α_4 and that specifically recognizes integrin β_7 being selected, said hybridoma being injected intraperitoneally into small mammals that are compatible with it and the monoclonal antibodies that are produced from said ascites being separated and purified.

[Claim 4] A method for the production of monoclonal antibodies as described in Claim 3 in which the human B-cell lymphoma is RPMI8866.

[Detailed Description of the Invention]

[0001]

[Field of industrial application] This invention relates to monoclonal antibodies that specifically recognize human integrin β_7, a hybridoma that produces them and a method for the production of said antibodies.

[0002]

[Prior art] Integrin α_4 β_1 (VLA-4) is expressed for the most part in monocytes. It infiltrates sites of inflammation through the agency of bonds with VCAM-1, which is a cell adhesion protein, and causes an inflammatory response. Consequently, impeding adhesion of VLA-4 and VCAM-1 is considered to be important in treatment in diseases such as asthma, allergies, arthritis and arteriosclerosis (M. Bosco and C. Chan, et al., J. Biol. Chem., Vol. 267, p. 8366, 1992).

[0003] VLA-4 belongs to the integrin β_1 subfamily and it is comprised of a heterodimer of integrin α_4 and integrin β_1. However, it has been reported that the integrin α_4 subunit is associated not only with integrin β_1, but also, in humans with integrin β_7 (M. Bosco, C. Chan et al., J. Biol. Chem., Vol. 267, p. 8366, 1992 , and David J. Erle et al., J. Biol. Chem., Vol. 266, p. 11009, 1991).

[0004] It has been reported that human integrin $\alpha_4 \beta_7$, lik $\alpha_4 \beta_1$, causes adh sion of lymphocyt s t endothelial cells through the agency of fibronectin and VCAM-1 (Curzio Ruegg, et al., J. Cell Biol., Vol. 117, p. 179, 1992).

[0005] Although various antibodies against integrin subunits are known, monoclonal antibodies against human integrin β_7 are not known.

[0006]

[Problems the invention is intended to solve] This invention has the objective of providing monoclonal antibodies that can bond specifically with integrin β_7, a hybridoma that produces them and a method for the production of said antibodies.

[0007]

[Means for solving the problems] The inventors conducted various studies, and, as a result, perfected this invention by discovering monoclonal antibodies that specifically recognize human integrin β_7 and a hybridoma that produces monoclonal antibodies that specifically recognize human integrin β_7.

[0008] We shall now present a detailed description of this invention.

[0009] The monoclonal antibodies of this invention and the hybridoma that produces them can be obtained by the following method of production.

(Preparation of antigen) In this invention, a purified hybridoma of human integrin $\alpha_4 \beta_7$ is used for the antigen that is employed for immunization of mammals.

[0010] The hybridoma of human integrin $\alpha_4 \beta_7$ can be obtained as d scribed below.

[0011] First, cells that express human integrin α_4 but that do not express human integrin β_1 are selected by the flow-cytometry analysis method from human lymphocytes selected from human memory T-cells, human monocytes and B-cell lymphoma using anti-human integrin α_4 antibodies (SG/17, acquired from the Department of Immunology, Faculty of Medicine, Shuntendo University) and anti-human integrin β_1 antibodies (SG/19, acquired from the Department of Immunology, Faculty of Medicine, Shuntendo University) (it being thought that, with the cells that are selected, the human integrin α_4 subunit and the human integrin β_7 subunit form a heterodimer). Next, the selected cells are solubilized with a solubilizable buffer that contains a nonionic surfactant and are passed through an immunoaffinity column in which the anti-human integrin α_4 antibodies (SG/17) are immobilized, with a heterodimer of human integrin α_4 β_7 being caused to adhere to said column, after which elution is performed with an acid, and a fraction containing the heterodimer of human integrin α_4 β_7 is obtained. Finally, the fraction that has been obtained is dialyzed overnight in distilled water, after which it is freeze-dried.

[0012] RPMI8866 (acquired from the Department of Immunology, Faculty of Medicine, Shuntendo University), which is a human B-cell lymphoma, is an example of cells that are selected by the aforementioned flow-cytometry method.

[0013] (Production of the hybridoma) The production of the hybridoma can be performed by a standard method as described below. Specifically, the freeze-dried product (antigen) of human integrin α_4 β_7 that has been obtained as

described above is dissolved in a phosphate-buffered physiological saline solution and the solution is administered to a small mammal, such as a hamster, with said animal being immunized, after which its spleen is xcised and antibody-producing spleen cells are prepared.

[0014] The antibody-producing spleen cells of the immunized animals are subjected to cell fusion with myeloma cells. The myeloma cells that are used may originate, for example, from mice. Cell fusion can be performed, for example, by the method of Milstein et al. (C. Milstein, et al., Nature, Vol. 256, p. 495, 1975). Specifically, it can be performed by reaction for approximately 1 to 3 minutes at 30°C to 40°C using 30% to 60% polyethylene glycol (average molecular weight of 1000 to 4000).

[0015] The monoclonal antibodies that are obtained in this way and that are produced by the hybridoma are screened by the flow-cytometry analytical method and a hybridoma that produces monoclonal antibodies that do not recognize human integrin α_4 and that specifically recognize human integrin β_7 are selected.

[0016] The hybridoma that was obtained as described above was designated as " a hybridoma producing anti-human integrin β_7 monoclonal antibody (TN114)." It was deposited in the Biological and Industrial Technology Institute of the Agency of Industrial Science and Technology [Deposit No., Industrial Technology Institute Microorganism Deposit No. 13202 (FERM P-13202)].

[0017] (Production f monoclonal antibody) Th monoclonal antibodies of this inv ntion can be obtained by injecting the hybridoma that is obtained as described above intraperitoneally into a small animal that is compatible with it, for example, a hamster, in which it is caused to proliferate and by separating and purifying the monoclonal antibodies of this invention from the ascites by a standard method.

[0018] The monoclonal antibodies (TN114) of this invention are obtained by using the "hybridoma producing anti-human integrin β_7 monoclonal antibody (TN114)" as the hybridoma.

[0019]

[Effect of the invention] Human integrin β_7 in human tissues can easily be detected by using the monoclonal antibodies of this invention. In addition, human integrin β_7 can be separated and purified from human tissues by using the monoclonal antibodies of this invention. Consequently, the monoclonal antibodies of this invention can be employed as reagents for detecting human integrin β_7 and as reagents for its separation and purification.

[0020] The monoclonal antibodies (TN114) of this invention control adhesion of human B-cell lymphoma (RPMI8866) to fibronectin (see Experimental Example 1), for which reason it is anticipated that they can be used as treatment agents for inflammatory diseases that are related to cell adhesion proteins such as fibronectin.

[0021] Experim ntal Example 1

Inhibitory action on adh sion of human B-cell lymphoma (RPMI8866) to fibronectin by means of anti-human integrin β_7 monoclonal antibodies:

(1) Test sample

Monoclonal antibodies of this invention (TN114; see Example 2)

[0022] (2) Experimental method

10 μg/ml human plasma fibronectin (manufactured by GIBCO) dissolved in 50 μl of phosphate-buffered physiological saline solution (pH 7.4, abbreviated hereafter as PBS) was poured individually into each well of a 96-well microtiter plate and the materials were cultured at 4°C. The solution in each well was removed and washed in PBS, after which blocking was performed for 2 hours with PBS containing 1% bovine serum albumin. Following that, it was washed 3 times with PBS and the bovine serum albumin was removed.

[0023] Further, the human B-cell lymphoma (RPMI8866) was cultured for 30 minutes together with a dimethyl sulfoxide solution (manufactured by Dojin Chemicals) of 10 μM 2',7'-bis(carboxyethyl)carboxyfluorescein tetraacetatoxy methyl ester (hereafter abbreviated as BCECF-AM), after which it was washed three times with serum-free lymphocyte culture medium (AIM V™ culture medium manufactured by GIBCO). The human B-cell lymphoma (RPMI8866) in which BCECF-AM was inc rporated that was obtained and the test sample were subjected to preliminary culture for 30 minutes, after which the sample was

poured individually into the wells of the aforementioned 96-well microtiter plate (1 x 10^5 of said cells per well; sample concentration: 20 μg/ml). The materials were cultured for 10 minutes at 37°C, after which each well was filled with PBS and was hermetically sealed with plate seal (manufactured by the Dainippon Pharmaceutical Company). Said plate was inverted and centrifugation was performed for 2 minutes at 800 rotations/minute. The solution in each well was removed, after which 100 μl of 1% Nonidet P-40™ (manufactured by Kanaraitech* were added and the cells remaining due to adhesion were dissolved. Next, the quantity of BCECF-AM in each well was determined using a fluoroscanner and the adhesion rate of the human B-cell lymphoma (RPMI8866) to the fibronectin was found taking this quantity as the index. The adhesion rate was calculated taking fluorescence intensity when the aforementioned experiment was conducted using a well not coated with fibronectin as 0% and taking the fluorescence intensity when human B-cell lymphoma (RPMI8866) (1 x 10^5 cells) in which the aforementioned BCEF-AM was incorporated was dissolved in 100 μl of 1% Nonidet P-40™ as 100%.

[0024] As a controls, the adhesion rates when anti-human integrin β_1 antibodies (SG/19), and anti-human integrin α_4 antibodies (SG/73, acquired from the Department of Immunology, Faculty of Immunology, Shuntendo University) were added and when antibodies were not added were found in the same way as described above.

* Phonetic transliteration of company name—Trans. note.

[0025] (3) Experimental Results

The results are shown in Figure 1. As should be evident from Figure 1, it was ascertained that the monoclonal antibodies inhibit adhesion of human B-cell lymphoma (RPMI8866) to fibronectin.

[0026]

[Examples] We shall now describe this invention by presenting examples.

[0027] In the examples, culture media (1) through (3) indicated below were used, depending on the objectives.

(1) RPM11640 culture medium

(2) Culture medium for cells (myeloma cells or hybridoma)

A culture medium obtained by adding the substances indicated below to culture medium (1) as described above

10% fetal calf serum

2mM L-glutamine

50 μM 2-mercaptoethanol

100 U/ml penicillin G

100 μg/ml streptomycin

20 mM sodium hydrogen carbonate

(3) HAT cultur medium

A culture medium obtained by adding the following substances to culture medium (2) as described above

0.1 mM hypoxanthine

0.4 μM aminopterin

16 μM thymidine

[0028] Example 1

Hybridoma that produces monoclonal antibodies that specifically recognize human integrin β_7

(Preparation of antigen) Various human lymphocytes (6×10^5 cells) were divided into three groups and each group was suspended in 50 μl of PBS. Anti-human integrin α_4 antibodies (SG/17) were added to one of these groups, anti-human integrin β_1 antibodies (SG/19) were added to another of these groups and nothing was added to the remaining group. These materials were cultured for 30 minutes at 4°C. After culture, they were washed with PBS, after which they were suspended in 50 μl of PBS containing 0.5 μl of goat anti-mouse IgG-FITC (manufactured by Olympus). Next, they were again cultured for 30 minutes at 4°C and were then washed with PBS, after which they were again suspended in 200 μl of PBS. Each suspension was analyzed by flow cytometry, cells that

expressed human integrin α_4 and that did not express human integrin β_1 were selected and RPMI8866, which is a human B-cell lymphoma, was obtained.

[0029] The RPMI8866 (3×10^8 cells) that was obtained was solubilized with 30 ml of solubilization buffer [50 mM tris-hydrochloric acid, pH 7.6, 150 mM sodium chloride, 1% polyoxyethylene (10) octylphenyl ether, 50 mM iodoacetamide, 2 mM magnesium chloride, 2 mM calcium chloride, 0.1% sodium azide, 1 mM phenylmethylsulfonyl fluoride] and was adsorbed to an immunoaffinity column in which anti-human integrin α_4 antibodies (SG/17) were bonded to cellulose beads. Next, it was eluted with 0.1 N glycine-hydrochloric acid buffer (pH 3.0) and a fraction containing a heterodimer of human integrin α_4 β_7 was obtained. The fraction that was obtained was dialyzed overnight in distilled water, after which it was freeze dried (yield, 10 μg). Said freeze-fried product was subjected to SDS-polyacrylamide gel electrophoresis, and the presence of human integrin α_4 β_7 was confirmed at 120-150 kDa.

[0030] (Production of hybridoma) 10 μg of the freeze-dried product of the α_4 β_7 heterodimer that was obtained as described above were suspended in 0.5 ml of PBS and Armenian hamsters were immunized by a 5x intraperitoneal injection at intervals of 1 to 2 weeks.

[0031] The spleen of the hamster was excised four days after the final immunization and antibody-producing spleen cells were prepared. Next, said spl en cells and mouse myeloma cells P3 x 63Ag8U.1 (ATCC CRL 1597) were mixed in a ratio of 5 : 1 and cell fusion was effected by reacting them for 2

minutes at 37°C using 50% polyethylene glycol (average molecular weight, 4000).

[0032] Cells that had undergone cell fusion were implanted in a 96-well microtiter plate and were cultured in HAT culture medium for 7 to 14 days at 37°C and in the presence of 5% carbon dioxide.

[0033] Next, the culture supernatant of implanted cells was screened by flow-cytometry analysis. The details of the flow-cytometry analysis method are described below.

[0034] 160 μl of human integrin β_7 hybridoma culture supernatant were divided into two parts. Half was cultured with 2×10^5 RPMI8866 cells and the remaining half was cultured with 2×10^5 Ramos cells (ATCC CRL 1596), with culture being performed for 30 minutes at 4°C. The cells were washed with PBS, after which the cells were resuspended for 30 minutes at 4°C in 50 μl of cell culture medium containing 0.5 μl of goat anti-hamster-IgG-FITC (manufactured by CALTAG) and were again washed with PBS. These cells were then resuspended in 200 μl of cell culture medium and were analyzed by flow cytometry.

[0035] As a result, it was ascertained that the four clones exhibited positive responses to the target antigens.

[0036] The four clones that were obtained in the aforementioned screening process were pr pared in amounts of 1/0.2 ml in cell culture medium

containing thymus cells (approximately 1×10^6 cells/ml) of BALB/c mice and were implanted in individual wells of a 96-well microtiter plate. Culture was performed at 37°C in the presence of carbon dioxide, after which colonies that could b discerned with the unaided eye were formed in 7 to 14 days. The colonies that were obtained were screened and cloned in the same way as described above, and, finally, confirmation procedures were performed by Western blotting using rabbit antiserum to β_7 peptides (acquired from Dr. Michael B. Brenner of Harvard Medical School in the United States), with a single clone strain that specifically recognizes human integrin β_7 being obtained (staining being found in the site of the 120 kD β-chain).

[0037] The single strain that was obtained was designated as "hybridoma producing anti-human integrin β_7 monoclonal antibody (TN114)" and was deposited in the Biological and Industrial Technology Institute of the Agency of Industrial Science and Technology [Deposit No., Industrial Technology Institute Microorganism Deposit No. 13202 (FERM P-13202)].

[0038] Example 2

Monoclonal antibodies that specifically recognize human integrin β_7: The hybridoma producing anti-human integrin β_7 monoclonal antibody (TN114) (1×10^7 cells) obtained in Example 1 are inoculated intraperiteroneally into BALB/c mice (3 animals) that had been given intraperitoneal administrations of 0.5 ml of 2,6,10,14-tetramethylpentadecane (manufactured by Nakarai Tesuku) in

advance. After about 1 week, ascites (approximately 3 ml/mouse) containing hamster anti-human integrin β_7 monoclonal antibodies (TN114) was obtained.

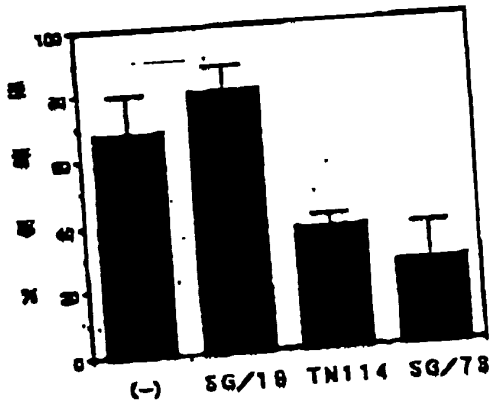
[0039] Next, 20 ml of PBS were added to approximately 10 ml of the ascites that was obtained and dialysis was carried out overnight with PBS at 4°C. This was passed through a 0.2-μm filter, after which separation and purification were carried out with a protein G Sepharose 4-fast flow column (manufactured by Pharmacia). Dialysis was again performed overnight with PBS at 4°C, with 5 ml of PBS solution of hamster anti-human integrin β_7 monoclonal antibodies (TN114) being obtained (concentration: 100 μg/ml).

[Brief Explanation of the Figure]

[Figure 1] This is a figure showing that the monoclonal antibodies (TN114) of this invention inhibit adhesion of human B-cell lymphoma (RPMI8866) to fibronectin.

[Figure 1]

[y- axis]: Adhesion rate %



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